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Cardiac Metallothionein Induction Plays the Major Role in the Prevention of Diabetic Cardiomyopathy by Zinc Supplementation

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Background—Our previous studies showed that transgenic mice that overexpress cardiac-specific metallothionein (MT) are highly resistant to diabetes-induced cardiomyopathy. Zinc is the major metal that binds to MT under physiological conditions and is a potent inducer of MT. The present study therefore explored whether zinc supplementation can protect against diabetic cardiomyopathy through cardiac MT induction.

Methods and Results—Diabetes was induced in mice (C57BL/6J strain) by a single injection of streptozotocin. Half were supplemented intraperitoneally with zinc sulfate (5 mg/kg) every other day for 3 months. After zinc supplementation, mice were maintained for 3 more months and then examined for cardiomyopathy by functional and morphological analysis. Significant increases in cardiac morphological impairment, fibrosis, and dysfunction were observed in diabetic mice but not in diabetic mice supplemented with zinc. Zinc supplementation also induced a significant increase in cardiac MT expression. The role of MT in cardiac protection by zinc supplementation was examined in cultured cardiac cells that were directly exposed to high levels of glucose (HG) and free fatty acid (FFA) (palmitate), treatment that mimics diabetic conditions. Cell survival rate was significantly decreased for cells exposed to HG/FFA but did not change for cells exposed to HG/FFA and pretreated with zinc or low-dose cadmium, each of which induces significant MT synthesis. When MT expression was silenced with the use of MT small-interfering RNA, the preventive effect of pretreatment with zinc or low-dose cadmium was abolished.

Conclusions—These results suggest that the prevention of diabetic cardiomyopathy by zinc supplementation is predominantly mediated by an increase in cardiac MT. (***Circulation***. 2006;113:544-554.)

Key Words: cardiomyopathy ∎ diabetes mellitus ∎ myocardium ∎ nutrition ∎ prevention

Diabetic cardiomyopathy has become a major cause of disability and mortality for diabetic patients.[1,2] Diabetic cardiomyopathy can occur clinically without major vascular disease, suggesting a primary role for direct effects of diabetes on cardiac myocytes.[1,2] We have demonstrated that exposure of cardiac cells to high levels of glucose (HG) caused a significant increase in cardiac cell death and accumulation of reactive oxygen species,[3] suggesting that diabetic cardiomyopathy may be directly related to diabetes-derived oxidative stress.[3,4] Antioxidant therapy for diabetic cardiovascular diseases has thus been extensively explored for the prevention of diabetic cardiomyopathy, but results are inconsistent because of factors such as difficulty in maintaining a consistent circulating antioxidant level, inadequate tissue distribution, and lack of suitable exogenous antioxidants.[4–6] Therefore, a strategy to enhance an endogenous and more efficient and nonspecific antioxidant may be a more effective approach to preventing diabetic cardiotoxicity.

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Metallothionein (MT) fulfills such criteria. MT is a cysteine-rich protein that binds metals such as zinc (Zn) and copper (Cu) and acts as an antioxidant that is very efficient in scavenging or quenching various free radicals or reactive oxygen species.[7,8] With the use of a cardiac-specific, MT-overexpressing transgenic (MT-TG) mouse model, MT was found to significantly protect from a variety of oxidative stimuli.[9–11] More importantly, MT was shown to significantly prevent diabetic cardiomyopathy and significantly suppress oxidative damage in the streptozotocin-induced type 1 diabetic mouse model.[12–14] MT was also implicated in the prevention of diabetic cardiotoxicity in a genetically predisposed type 1 diabetic mouse model.[15,16] Taken together, these results suggest that MT would be a candidate for antioxidant therapy to prevent diabetic cardiomyopathy.

Development of a pharmaceutical approach for upregulating endogenous MT to prevent diabetic cardiomyopathy is

feasible because MT exists in various tissues, including the mammalian heart, and is also highly inducible.[7,8] Results from chemically induced MT synthesis in various organs have indicated significant protection from oxidative damage against radiation and chemicals.[7,8] Cardiac MT induction that prevents cardiac damage from various oxidative stimuli has also been documented extensively.[7,17,18] Because MT primarily binds to Zn under physiological conditions, Zn has been shown to be an effective inducer of MT synthesis in the heart that can provide effective protection against oxidative damage.[17,18] In addition, that MT upregulation in response to Zn supplementation prevents various pathogeneses has been documented in humans.[19,20]

The present study therefore was undertaken to investigate the protective effects of Zn supplementation, with cardiac MT induction, on diabetic cardiomyopathy as a possible pharmaceutical approach to prevention of diabetic cardiovascular complications.

Methods

Diabetic Mouse Model

Male C57BL/6J mice, 8 weeks of age, obtained from Harlan Bioproducts for Science, Inc (Indianapolis, Ind), were housed in the University of Louisville Research Resources Center at 22°C with a 12-hour light/dark cycle and free access to rodent chow and tap water. All animal procedures were approved by the Institutional Animal Care and Use Committee, which is certified by the American Association for Accreditation of Laboratory Animal Care. Mice were divided into nondiabetic and diabetic groups (n=60 in each group). Diabetic mice were induced by intraperitoneal injection of a single dose of streptozotocin (150 mg/kg body wt; Sigma Chemical Co) dissolved in a sodium citrate buffer (pH 4.5). Whole blood obtained from each mouse's tail vein was used for glucose monitoring with the use of a SureStep complete blood glucose monitor (LifeScan). Streptozotocin-treated mice with whole-blood glucose levels >12 mmol/L, examined on day 3 after streptozotocin treatment, were considered diabetic. Mice serving as vehicle controls (nondiabetic mice) were given the same volume of sodium citrate as described previously.[3]

Supplementation With Zn

On the day after streptozotocin-injected mice were diagnosed as diabetic, both nondiabetic and diabetic mice were randomly divided into 2 groups (n=30 for each): one as control, the other to receive Zn supplementation. Zn supplementation was given by intraperitoneal injection with 5 mg Zn per kilogram body weight every other day for 3 months. Chronic supplementation with Zn to induce cardiac MT has not been reported previously; however, acute or subacute doses of Zn over a range of 5 to 20 mg/kg for rats and mice have been documented.[17,18,21] Therefore, we selected the relatively low dose of Zn (at 5 mg Zn per kilogram) given every other day to avoid excess free Zn accumulation in the tissues. Zn supplementation for 3 months was selected because we found that diabetic mice 3 months after induction of hyperglycemia started to show cardiac dysfunction by echocardiography and other assessments.[22]

Among the nondiabetic mice, 5 Zn-supplemented and 5 non–Zn-supplemented mice were euthanized at 1 month and 3 months after Zn supplementation to harvest the heart and liver for measurement of cardiac and hepatic MT contents and hepatic mineral contents.

Measurements of Serum Triglyceride

Whole blood was collected from the dorsal vena cava of the anesthetized animals, and serum was prepared with the use of a serum separator apparatus (Becton Dickinson) to measure the triglyceride level following the instructions provided in the corresponding kits (Sigma Chemical Co).

Assessment of Left Ventricular Performance

Cardiac performance was assessed with the use of left ventricular (LV) hemodynamic analysis as described previously.[22,23] Mice were anesthetized with tribromoethanol (0.5 mg/g body wt, by 0.25 mL/g IP injection). A midline incision (1 to 2 cm) in the throat area external to the trachea was made for the insertion of a PE-100 catheter to ensure a patent airway. The common right carotid artery was isolated, and a small incision was then made in the artery for the insertion of a hand-stretched PE-50 catheter that was connected to a transducer to transform the signals to the Heart Performance Analyzer TM 400 (Micro-Med, Inc). The frequency response of this system is 45 cycles per second after amplification of transducer microvoltage, followed by microprocessor sampling at 1000 points per second, which is suitable to measure heart rate from 12 to 999 bpm and maximum dP/dt (dP/dt_{max}) from 100 to 65 500 mm Hg/s.

While the pressure tracing was monitored, the catheter was advanced slowly via the common carotid artery into the ascending aorta and retrogradely into the LV. Arterial blood pressure was measured before catheter insertion into the LV. When a waveform characteristic of LV pressure was achieved, the wound was covered to minimize liquid evaporation. The animal was then allowed to stabilize for 20 to 30 minutes before the LV pressure tracing was recorded at baseline and after catecholamine stimulation with isoproterenol (delivered via the femoral vein at 0.8 ng/g per minute for 4 minutes). Myocardial functional changes in response to isoproterenol were recorded 1 minute after isoproterenol infusion. At the end of each experiment, the chest was opened to confirm proper catheter position.

Histopathological Examination by Light and Electron Microscopy

Heart tissues were cut into ≈3.0-mm–thick slices and fixed with 10% neutral formalin overnight. The tissue slices were embedded in Paraplast. Three sections of 4-μm thickness per heart were prepared and stained with hematoxylin and eosin. For electron microscopic examination, heart tissue was cut into small pieces and prefixed in 2.5% glutaraldehyde (0.2 mol/L cacodylate buffer, pH 7.4) for 4 hours and postfixed in 1% buffered osmium tetroxide for 1 hour and embedded in Epon 812. Ultrathin sections were examined with a JEM-1200 EX electron microscope, as described previously.[9,11] Semiquantitative analysis for cardiac morphological changes under light and electron microscopes was performed on the basis of a 1 to 4 scoring method, as described previously.[15,24,25]

Cardiac fibrosis was determined by staining 4-μm sections for collagen (0.1% Sirius red/F3BA) as described in our previous study.[14] For MT expression, cardiac sections were stained as described previously.[22] The Sirius red–stained sections were assessed for the proportion of fibrosis (collagen) in the heart tissues by semiquantitative analysis on the basis of a 1 to 4 scoring method, as described previously.[24,25]

Detection of Connective Tissue Growth Factor, MT, and Superoxide Dismutase Expression by Western Blotting

Cardiac tissues were homogenized with lysis buffer containing 50 mmol/L Tris-HCl (pH 7.5), 5 mmol/L EDTA, 10 mmol/L EGTA, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin A, and 50 μg/mL phenylmethylsulfonyl fluoride. Tissue proteins were collected by centrifuging at 12 000 rpm at 4°C in a Beckman GS-6R centrifuge for 10 minutes. The protein concentration was determined, and the sample was then mixed with loading buffer (40 mmol/L Tris-HCl, pH 6.8, 1% SDS, 50 mmol/L dithiothreitol, 7.5% glycerol, 0.003% bromophenol blue) and heated at 95°C for 5 minutes and then subjected to electrophoresis on a 16% SDS-PAGE gel at 120 V. After electrophoresis of the gel, the proteins used for connective tissue grow factor (CTGF) and superoxide dismutase (SOD) analysis were transferred to a nitrocellulose membrane in transfer buffer containing 20 mmol/L Tris, 152 mmol/L glycine, and 20% methanol. Transfer of proteins for MT analysis was done in the above buffer modified by addition of 2 mmol/L $CaCl_2$. The membranes were

rinsed briefly in PBS and blocked in blocking buffer (5% milk and 0.5% BSA) at room temperature for 2 hours. The membranes were incubated with goat anti-CTGF polyclonal antibody (1:500 dilution), rabbit anti–Cu/Zn-SOD polyclonal antibody (1:1000 dilution), or anti-MT polyclonal antibody (1:1000 dilution) (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), respectively, for 2 hours at room temperature. Membranes were then washed 3 times with TBS-T containing 0.05% Tween-20 and reacted with secondary horseradish peroxidase–conjugated antibody for 1 hour. Antigen-antibody complexes were visualized with the use of an enhanced chemiluminescence (ECL) kit (Amersham). Actin expression was used as a control. After detection of CTGF and SOD, the membranes were stripped with stripping buffer (50 mmol/L Tris-HCl, pH 7.4, with 150 mmol/L NaCl and 0.1% β-mercaptoethanol) for 1 hour at room temperature, incubated with monoclonal anti-actin antibody (Sigma Chemical Co, St Louis, Mo) at a concentration of 1:1000 for 1 hour, and developed for chemiluminescent signals with the ECL kit. However, since the transfer conditions (with $CaCl_2$) for MT protein analysis were not appropriate for detection of actin, when MT analysis was performed, 2 parallel gels were run under the same conditions except that 1 gel (MT gel) was transferred to a membrane in transfer buffer containing $CaCl_2$, while the other (actin gel) was transferred to another membrane in buffer without $CaCl_2$. Western blotting was performed on the 2 membranes separately, with actin blotting used only to confirm equal loading of protein. Data analysis of MT blots was therefore based on the fold difference relative to control (in the same membrane) rather than on the fold difference relative to actin (in the other membrane).

Metal Concentrations in the Livers

Hepatic mineral concentrations, including Zn and Cu, were determined with the use of inductively coupled argon plasma emission spectroscopy (model 35608, Thermo ARL-VG Elemental) after lyophilization and digestion of the tissues with nitric acid and hydrogen peroxide.[23] Metal concentrations were expressed as $\mu g/g$ dry tissue.

Cell Cultures and Treatments

To define the role of MT in cardiac protection by Zn supplementation, a cell culture model was used in which cardiac cells were first subjected to Zn pretreatment and then directly exposed to HG and free fatty acid (FFA) to mimic diabetic conditions. H9C2 cells (ATCC CLR-1446; Rockville, Md) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, and 100 U/mL penicillin at 37°C in a humidified chamber containing 5% CO_2. When cell populations reached 40% to 50% confluence, the cultures were exposed to D-glucose (Sigma Chemical Co) in a final concentration of 22.5 mmol/L as HG and to FFA (palmitate bound to BSA at a ratio of 5:1) in a final concentration of 50 $\mu mol/L$ for 24 hours. In addition, some cultured cells were exposed to 5.5 mmol/L D-glucose as controls. After treatments, the monolayer cultures were collected

with the use of a gum rubber scraping device and lysed by lysis buffer. Zn pretreatment was performed by exposing cells to 50 $\mu mol/L$ Zn for 24 hours to induce MT synthesis and then incubating with fresh medium without Zn for another 48 hours before exposure to HG/FFA. In addition, to exclude a direct protective role of Zn, low-dose cadmium (Cd) instead of Zn was used as a MT inducer in some experiments.

Analysis for Cell Viability

Cell viability was determined by a short-term microculture tetrazolium (MTT) assay. In a 96-well microplate, 2.5×10^4 cells per well were incubated in 100 μL of culture media and exposed to different concentrations of glucose for varying time periods. The media were removed and replaced with 90 μL of DMEM (containing no phenol red or FBS) and 10 μL of MTT solution (2 mg/mL phosphate buffer) for 4 hours. After the MTT-containing DMEM was removed, the remaining formazan blue crystals were dissolved in 75 μL of 0.04N HCl/isopropyl alcohol solution. Absorbance at 540 nm was measured with a microplate reader (model FL 311; Bio-Tek Instruments), as described previously.[3]

Analysis of Cardiac Cell MT and SOD Protein Expression

For cardiac cell MT or SOD protein expression, these cells were lysed with lysis buffer containing 2% SDS, 10% glycerol, and 62.5 mmol/L Tris (pH 7.0) and then sonicated. Cell proteins were collected by centrifuging at 12 000 rpm at 4°C in a Beckman GS-6R centrifuge for 10 minutes. Protein determination and Western blotting procedures were the same as those used for cardiac tissue MT and SOD expression, as described above.

Measurement of MT mRNA by Northern Blotting

Total cellular RNA was extracted from cultured cells with the use of the Trizol reagent. Random primed first-strand cDNA was prepared from total cellular RNA with the use of Superscript II and amplified by polymerase chain reaction (PCR) using rat MT I primers (sense primer, 5′-TCGCTTACACCGTTGCTCCA-3′; anti-sense, 5′-CTCTGAGTTGGTCCGGAAATTATT-3′). The purified PCR fragment was subcloned into a PCR II vector as a template for synthesis of the cRNA probe. The plasmid was linearized with Bgl II, and the antisense cRNA probe was produced with the use of the T7 RNA polymerase (Maxiscript kit from Ambion) in the presence of α-^{32}P UTP (Amersham). Total cellular RNA extracted from cultured cells (15 μg per lane) was fractionated by electrophoresis through a denaturing gel (0.66 mol/L formaldehyde in 1% agarose), downward transferred onto nylon membranes and vacuum-dried at 80°C for 2 hours. Membranes were hybridized to the antisense cRNA probes, as described above, in the hybridization solution (Ambion) at 68°C for 16 hours after a 1-hour prehybridization in the same solution. Membranes were then washed under high-stringency conditions
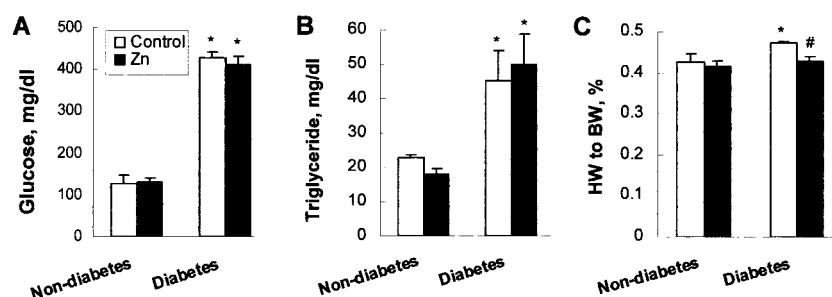
Figure 1. Blood glucose, serum triglycerides, and heart weight in mice after 6 months of streptozotocin-induced diabetes. Some mice were supplemented with Zn for 3 months immediately after the onset of diabetes (indicated by Zn). Both whole-blood glucose levels under fasting conditions (A) and serum triglycerides (B) were elevated by diabetes but not affected by Zn supplementation. Heart weight (HW) normalized to body weight (BW) (C) was increased by diabetes, an effect that was prevented by Zn supplementation. n=15 per group; *P<0.05 vs corresponding controls (nondiabetes without Zn supplementation); #P<0.05 vs diabetes without Zn supplementation.
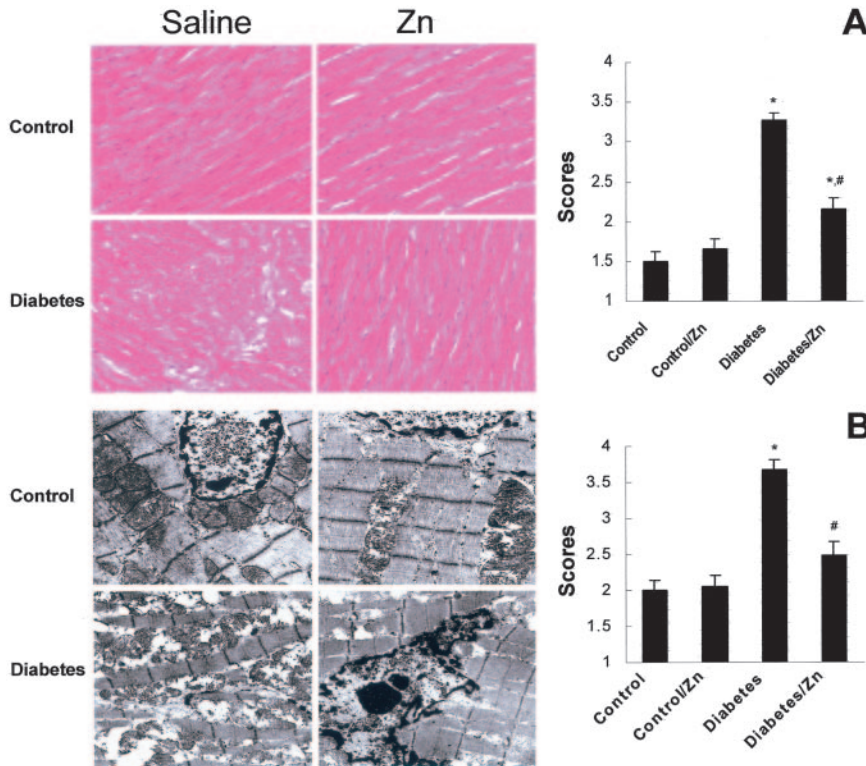
Figure 2. Effects of Zn on cardiac morphology of streptozotocin-induced diabetic mice 6 months after onset of diabetes. Semiquantitative scoring of pathology in hematoxylin and eosin–stained light micrographs (A) and in electron micrographs (B) indicated that diabetes enhanced morphological defects that were prevented when diabetic mice were supplemented with Zn. At least 5 mice in each group and 3 slides for each animal were analyzed. *$P<0.05$ vs corresponding controls; #$P<0.05$ vs diabetes without Zn supplementation.

(final wash $0.1\times$ SSC plus 0.1% SDS at 68°C) while monitored with a Geiger counter. Radioactivity was recorded on x-ray film.

Small-interfering RNA and Transfection

To determine whether MT expression plays a critical role in the preventive action of Zn or Cd pretreatment against HG/FFA-induced cytotoxicity, MT mRNA expression was silenced by incubation with MT small-interfering RNA (siRNA), as described previously.[14] siRNA transfections were performed with RNAiFect Transfection Reagent (Qiagen) according to the manufacturer's recommendations. For rat MT1 siRNAs, sense is 5′-CCCAGGGCUGUGUCUGCAAUU-3′ and antisense is 5′-UUGCAGACACAGCCCUGGGCA-3′. For control siRNA, sense is 5′-(UUCUCCGAACGUGUCACCU)dTdT-3′ and antisense is 5′- (ACGUGACACGUUCGGAGAA)dTdT-3′. All these sense and antisense sequences were designed and synthesized by Qiagen Inc.

Cells in 96-well plates were pretreated with Zn (50 μmol/L) or Cd (1.0 μmol/L) for 24 hours and then washed with PBS. MT1 siRNA (0.2 μg; final concentration, 1 μg/mL) or nonsilencing siRNA control (0.2 μg; Qiagen) in DMEM containing 10% FBS was added for 6 hours and then removed by washing with PBS. The medium was replaced with fresh DMEM containing 10% FBS, in which the cells were incubated for 42 hours, after which 22.5 mmol/L glucose and 50 μmol/L palmitate were added for 24 hours. Cell viability was assessed by MTT analysis.

After pretreatment with Zn or Cd for 24 hours, cells at 80% confluence in a 100-mm dish were transfected with 5 μg of MT1 siRNA and 5 μg of siRNA control (final concentration, 1 μg/mL) for 6 hours. RNA was extracted immediately after siRNA transfection by Trizol for Northern blot analysis, and protein was extracted at 48 hours after siRNA transfection for Western blot analysis.

Statistical Analysis

Data were described either by mean±SD for normally distributed variables or by median and range for skewed variables. For all statistical analyses, 1-way or 2-way ANOVA models were used for 1 (ie, time effect as indicated in Figure 4B, Figure 5A, and Figure 6B) or 2 responses (ie, diabetes and Zn supplementation, as shown in Figure 1 through Figure 7 and in part of the data in the Table). The

overall F test was performed to show the significance of the ANOVA models. The significance of the interactions and main effects was taken into consideration, and a proper method of pairwise comparison was chosen for each ANOVA.

The Tukey Studentized range test was used to test all pairwise comparisons among means. The Levene variance homogeneity test was applied to these laboratory variables. Because some variables seriously violated the assumptions for normality and homogeneity of variances, a significant difference was alternatively evaluated ($P<0.05$) with the Kruskal-Wallis test, finding those groups that were significantly different with the Mann-Whitney U test for 2 independent samples. To avoid an accumulation of errors due to multiple comparisons, the significance level was modified by dividing critical value ($P<0.05$) between the numbers of comparisons made (Bonferroni correction). SAS 9.1 (SAS Institute Inc) was used for all statistical tests.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Supplementation With Zn Prevented Diabetic Cardiomyopathy

We have previously demonstrated that MT-TG mice show a significant resistance to diabetes-induced cardiotoxicity.[12,13] The hearts of MT-TG mice contained 100-fold more cardiac MT and 3-fold greater cardiac Zn than wild-type mice, without changes of other metals such as magnesium, calcium, iron, and potassium (data not shown). These results suggested that enhancement of cardiac MT was related to an increase in Zn binding; therefore, we assumed that Zn may be an effective inducer of cardiac MT that could prevent diabetic cardiomyopathy. To test this hypothesis, streptozotocin-induced diabetic and nondiabetic mice were supplemented with Zn by intraperitoneal injection of 5 mg Zn per kilogram
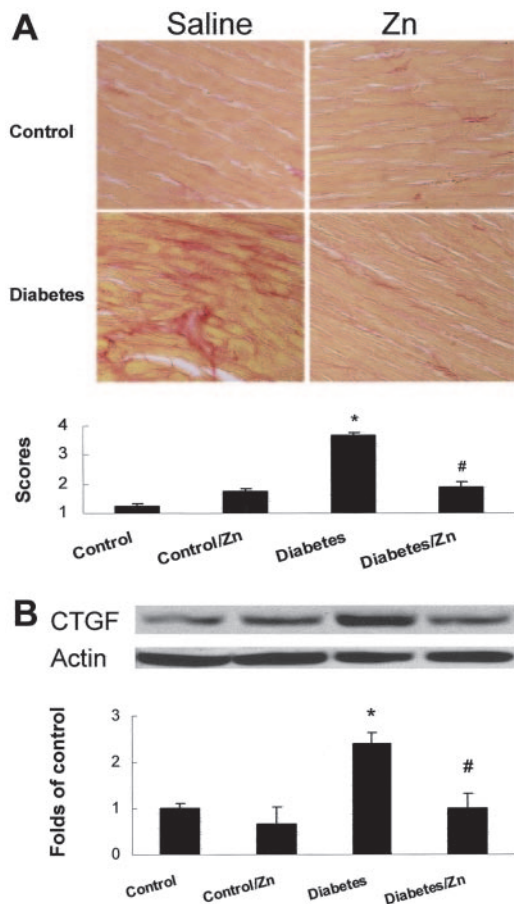
Figure 3. Effect of Zn supplementation on cardiac fibrosis in streptozotocin-induced diabetic mice determined 6 months after diabetes onset. Sirius red staining for collagen indicated an enhanced fibrosis in hearts of diabetic mice that was attenuated by Zn supplementation (A). Western blotting for CTGF, a critical upstream mediator of cardiac fibrosis, indicated an enhancement of CTGF expression with diabetes that was ameliorated by Zn supplementation (B). Three animals for the control/Zn group and at least 5 animals from each of the other groups were analyzed. *$P<0.05$ vs corresponding controls (nondiabetes without Zn supplementation); #$P<0.05$ vs diabetes without Zn supplementation.

body weight every other day for the first 3 months of a 6-month experiment. At the end of the experiment (6 months after diabetes onset), whole-blood glucose, serum triglycerides, and heart weight were measured. Diabetic mice showed consistent increases in fasting blood glucose and serum triglyceride levels for the entire period of 6 months compared with control (data not shown). Zn supplementation did not affect these measurements (Figure 1A and 1B). Diabetes caused significant increases in the ratio of heart weight to body weight that were prevented by Zn supplementation (Figure 1C).
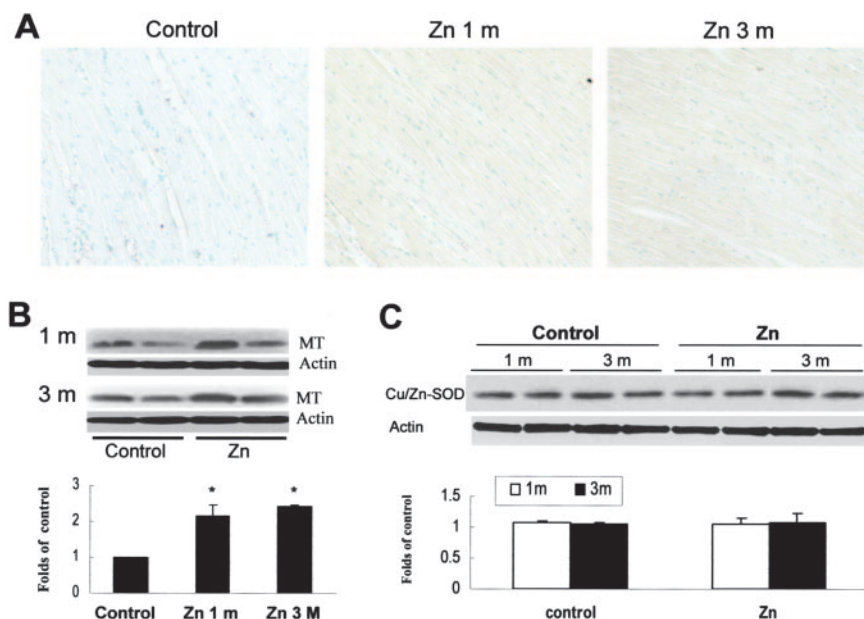
Effects of Zn supplementation on diabetic cardiomyopathy were determined at 6 months after diabetes onset by examination of morphological changes and development of fibrosis. Morphological observation by hematoxylin and eosin staining under light microscopy showed that diabetes induced a significantly disorganized cardiac architecture because of extensive interstitial fibrosis with mononuclear inflammatory

infiltration, changes that were not observed in the hearts of diabetic mice supplemented with Zn (diabetes/Zn in Figure 2A). Ultrastructural examination by electron microscopy demonstrated that control and control/Zn mice show normal myocardial fine structure, with myofibrils composed of regular and continuous sarcomeres demarcated by Z lines and adjacent myofibrils. Rows of moderately electron-dense mitochondria intervene between myofibrils. Diabetic myocardium shows randomly distributed mitochondria between poorly organized and disrupted myofibrils with increased myofibril spacing in an electron-lucent sarcoplasm, abnormalities that were significantly reduced in diabetes/Zn mice (Figure 2B).

As an index of the cardiac fibrotic effect of diabetes, Sirius red staining of collagen was used; fibrosis was found to be significantly increased in hearts of diabetic mice, an effect that was inhibited by Zn supplementation (Figure 3A). To further confirm the protective effect of Zn supplementation against fibrosis in diabetic hearts, cardiac expression of the critical fibrosis mediator CTGF[26,27] was examined by Western blotting. Cardiac CTGF expression was significantly increased in hearts of diabetic mice but not in hearts of Zn-supplemented diabetic mice (Figure 3B).

Cardiac function was evaluated by LV hemodynamic analysis in control and diabetic mice under resting and stress conditions 6 months after diabetes onset (Table). It was noted that certain functional parameters varied significantly and were not normally distributed. Our attempt to normalize these variables by logarithm transformation failed. Therefore, we were forced to use the nonparametric test. When multiple tests were performed, we used the sequential Bonferroni method to correct for an accumulation of errors. Basal heart rate in the diabetic mice was similar to that of control. However, enhancement of heart rate after stimulation with the β-adrenergic agonist isoproterenol was significantly less in diabetic animals than in control, an effect that was prevented by Zn supplementation (diabetes/Zn). No significant change for either systolic or diastolic blood pressure was observed in diabetic mice compared with control with or without Zn supplementation (Table).

The maximal rate of intraventricular pressure rise (dP/dt_{max}) was lower in the diabetic mice than in control, suggesting reduced LV contractility. In addition, there was slowing of LV relaxation in the diabetic mice compared with control, as indicated by the reduced minimum dP/dt and increased τ (the time constant of active relaxation). Supplementation with Zn improved both LV contraction and relaxation in the diabetic mice to near control levels. Furthermore, augmentation of dP/dt_{max} after β-adrenergic stimulation was markedly reduced in diabetic mice, indicating catecholamine desensitization. Zn supplementation markedly improved the catecholamine contractile response in diabetic mice. In addition, the slowing of LV relaxation in diabetic mice, shown by reduced dP/dt_{min} and increased τ, was also attenuated by Zn supplementation (Table). Thus, zinc improved both basal and stimulated LV systolic and diastolic function in diabetic hearts and improved inotropic reserve, consistent with the aforementioned improvements in cardiac morphology.

Figure 4. Cardiac MT protein synthesis induced by Zn supplementation for 1 or 3 months in nondiabetic mice. Immunohistochemical staining for MT protein indicates a qualitative increase in MT with Zn supplementation (A), a finding supported by Western blotting (B). Cardiac Cu/Zn-SOD as measured by Western blotting was not affected by Zn supplementation in nondiabetic mice (C). At least 5 mice per group were included. $*P<0.05$ vs corresponding controls.

Supplementation With Zn Induced Cardiac MT Synthesis

To determine whether Zn supplementation induces cardiac MT induction, cardiac MT protein was assessed. A significant increase in cardiac MT protein was initially observed by immunohistochemical staining in the hearts of mice with 1- and 3-month Zn supplementation (Figure 4A). The increased cardiac MT expression was further confirmed by Western blot assay (Figure 4B) showing ≈2- and 3-fold increases in the nondiabetic mice with 1- and 3-month Zn supplementation, respectively, compared with nondiabetic mice without Zn supplementation. To determine whether Zn supplementation also induces other antioxidants, Cu/Zn-SOD was examined by Western blot assay, which showed that cardiac Cu/Zn-SOD content was not significantly changed in nondiabetic mice in response to Zn supplementation for 1 or 3 months (Figure 4C).

Supplementation With Zn Induced Hepatic MT Synthesis and Mineral Changes

Because Zn supplementation constitutes a systemic exposure to Zn, MT protein level may be induced in multiple organs. A representative noncardiac tissue, the liver, was examined for MT induction with the use of Western blotting and showed a significant MT synthesis (Figure 5A). To explore the effect of Zn supplementation on alteration of organ metals, hepatic metal levels were measured. Hepatic metal content was measured because the mouse heart is too small to measure multiple parameters and because the liver is an organ that contains large quantities of Zn[28] and may thus be regarded as sensitive to changes in Zn status. Diabetes significantly decreased hepatic Zn levels at 1 and 3 months after diabetes onset (Figure 5B). Supplementation with Zn did not change hepatic Zn levels in nondiabetic mice but significantly increased hepatic Zn levels in diabetic mice at both 1 and 3 months after diabetes onset. Diabetes caused a significant decrease in hepatic Cu level, and Zn supplementation also

decreased hepatic Cu level in nondiabetic mice (Figure 5C). However, Zn supplementation did not further depress the diabetes-caused decrease in hepatic Cu and even increased hepatic Cu in the diabetic mice with 1-month Zn supplementation.

Preinduction of MT by Zn or Cd Significantly Protected Cardiac Cells From HG/FFA-Induced Cytotoxicity

The aforementioned animal experiments indicated that Zn supplementation provided significant prevention of diabetic cardiomyopathy, which is most likely attributed to Zn-induced cardiac MT induction; however, they were unable to exclude a direct role of Zn in this protective action against diabetic cardiomyopathy because Zn is also a potent antioxidant.[28] Therefore, in the following experiments, cultured cardiac cells exposed to conditions that mimic diabetes in vitro were used to determine the role of MT in the cardiac protection against diabetes by supplementation with Zn. We have demonstrated that diabetes caused both hyperglycemia and hyperlipidemia, as shown by an increase in serum triglyceride (Figure 1A and 1B). In addition, emerging evidence indicates that, in addition to glucotoxicity, lipotoxicity is also a critical mediator of diabetic cardiomyopathy. Therefore, cardiac cells (H9C2 cell line) were exposed to both HG and FFA (palmitate).

Exposure of cardiac cells to HG/FFA (22.5 mmol/L glucose/50 μmol/L palmitate) for 24 hours caused a significant decrease in cell viability, measured by the MTT assay (Figure 6A). To explore whether supplementation with Zn to these cells can prevent HG/FFA-induced cytotoxic effects, these cells were treated with 50 μmol/L Zn for 24 hours and then were further cultured for another 48 hours in fresh medium without Zn supplementation before exposure to HG/FFA. The time interval between Zn treatment and HG/FFA treatment is to avoid the direct interaction of remaining free Zn in medium with FFA. As shown in Figure 6A, the HG/FFA-induced
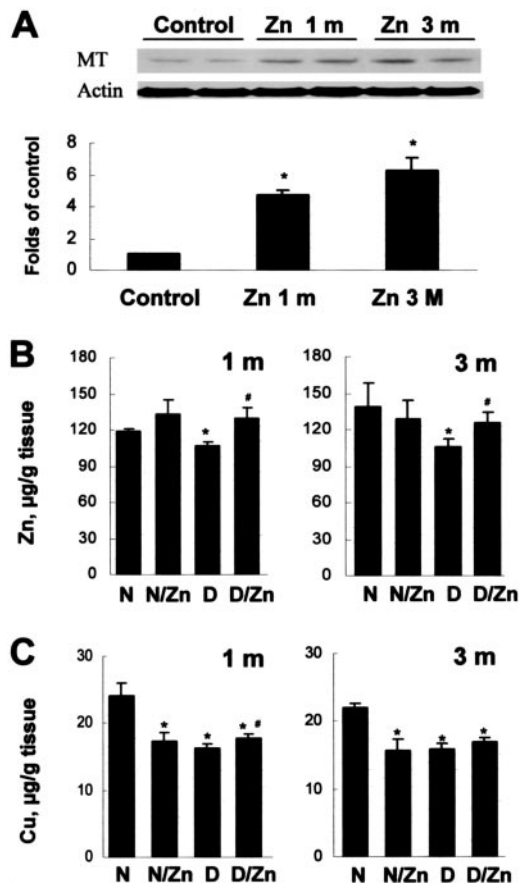
Figure 5. Effect of Zn supplementation on hepatic MT protein synthesis of nondiabetic mice and hepatic Zn and Cu levels of both nondiabetic and diabetic mice, measured at 1 or 3 months after diabetes onset. Zn supplementation elevated hepatic MT as determined by Western blotting (A). Hepatic Zn concentration was depressed by diabetes, an effect that was attenuated by Zn supplementation (B). Hepatic Cu concentration was depressed by either Zn supplementation or diabetes (C). Zn supplementation of diabetic mice somewhat attenuated the drop in Cu concentration caused by diabetes after 1 month (C). At least 5 mice for MT measurement (A) and 10 mice for Zn and Cu measurements (B and C) were included. N indicates control; N/Zn, control/Zn; D, diabetes; and D/Zn: diabetes/Zn. $*P<0.05$ vs corresponding controls; $\#P<0.05$ vs diabetes without Zn supplementation.

decrease in cell viability was significantly prevented by Zn pretreatment. The 24-hour Zn treatment also significantly induced MT mRNA transcription (Figure 6B) and protein synthesis (Figure 6D) but did not increase Cu/Zn-SOD content (Figure 6D). This suggests that the cells were protected from HG/FFA probably by Zn-induced MT.

To further show that MT plays the major role in the protective action of Zn supplementation, cardiac cells were preexposed to Cd, a nonessential trace metal as well as a well-known MT inducer, under the same conditions as Zn pretreatment. Pretreatment with Cd also provided significant cytoprotection and MT induction (Figure 6A, 6C, and 6D), as did Zn pretreatment. These results suggested that MT plays the major role, at least in Cd-pretreated cells, in the cytoprotection against effects of diabetes.

The direct role of MT in the cytoprotection by Zn or Cd pretreatment against diabetes was also further defined by silencing

MT transcriptional expression in the cells exposed to Zn or Cd by the MT siRNA strategy. Cardiac cells were treated with Zn or Cd for 24 hours, washed free of Zn or Cd, and transfected with MT siRNA for 6 hours. The MT transcriptional expression (Figure 7A) and translational expression (Figure 7B) were significantly inhibited by MT-specific siRNA but not by the siRNA control. Under the aforementioned experimental conditions, the protective action of pretreatment with Zn or Cd against HG/FFA-induced cytotoxicity was abolished by silencing of MT mRNA expression (Figure 7C). These results strongly indicate that the protective action of pretreatment with either Zn or Cd against the HG/FFA-induced cytotoxic effect is predominantly MT dependent.

Discussion

The present study explores the protective benefits of Zn supplementation in diabetes-induced cardiomyopathy. In the streptozotocin-induced type 1 diabetic mouse model, significant cardiomyopathy was observed, as characterized by significant increases in cardiac morphological abnormalities, cardiac fibrosis, LV systolic and diastolic dysfunction, and catecholamine desensitization. These changes were ameliorated by Zn supplementation, an intervention that also induced cardiac MT synthesis. In addition, examination of hepatic MT and minerals revealed that diabetes caused significant decreases in hepatic Zn and Cu and that supplementation with Zn improved both the hepatic Zn and, to a lesser extent, hepatic Cu content. With the use of cultured cardiac cells in combination with different MT inducers and a siRNA strategy to silence MT transcriptional and translational expression, the direct role of MT in the protection of Zn supplementation against HG/FFA cytotoxicity was confirmed.

Although multiple mechanisms are involved in the pathogenesis of diabetic cardiomyopathy, much evidence has indicated the important role of oxidative stress.[1,4] MT, as a potent antioxidant, protects against oxidative damage more efficiently than do other known antioxidants.[7,8,29] Our studies using cardiac-specific MT-TG mice have demonstrated that MT significantly prevented diabetes-derived superoxide accumulation and peroxynitrite-induced damage, leading to a significant prevention of diabetic cardiomyopathy.[12–14] These observations have been supported by others.[15,16] Such studies clearly indicate a great potential for the clinical application of MT in the prevention of diabetic cardiomyopathy.

Because there are still many unresolved issues for the clinical use of gene therapy, a suitable alternative may be to develop pharmaceutical MT inducers for the prevention of diabetic cardiomyopathy. Prior studies have shown that MT exists in many mammalian organs and is highly inducible by a variety of agents.[7,8] Because MT primarily binds to Zn under physiological conditions, Zn may be an effective candidate for inducing MT synthesis. Many studies have demonstrated that Zn supplementation provides effective protection in the heart against a variety of oxidative injuries.[7,17,18] In addition, the protective benefits of Zn supplementation have also been indicated for noncardiac systems of diabetic patients[30–32] and animals.[33] For instance, 6 weeks of Zn supplementation (660 mg/d) to diabetic patients was shown to significantly reduce the severity of peripheral neuropathy as assessed by motor nerve conduction velocity.[32] Additionally, in a rat model, diabetes-induced bone loss, as indicated by depression of calcium content, alkaline phosphatase
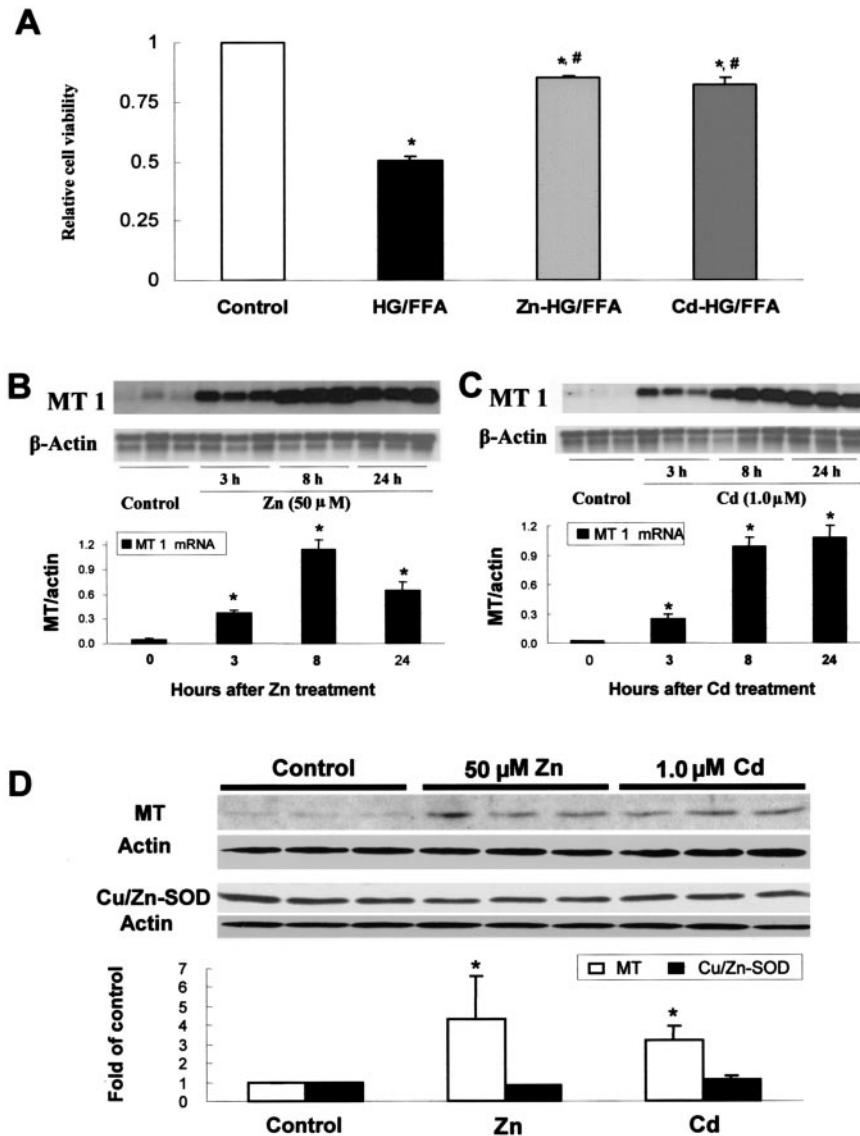
Figure 6. HG/FFA-induced cytotoxicity in cardiac cells and its prevention by Zn or Cd pretreatment, as well as MT induction by Zn or Cd. Cardiac cells (H9C2) exposed to HG/FFA for 24 hours exhibited a significant decrease in cell viability as measured by the MTT assay (A). HG/FFA-induced cytotoxicity was significantly prevented by 24-hour pretreatment with Zn (50 μmol/L) or Cd (1.0 μmol/L) (Zn-HG/FFA or Cd-HG/FFA in A). Zn or Cd pretreatment and HG/FFA treatment were separated by 48-hour intervals. Zn or Cd pretreatment significantly increased MT transcriptional (B or C) and translational (D) expression but did not increase Cu/Zn-SOD translational expression (D). Three experiments with at least 3 samples per group were performed. *$P<0.05$ vs control; #$P<0.05$ vs HG/FFA only. HG indicates 22.5 mmol/L glucose; FFA, 50 μmol/L palmitate bound to BSA in a 5:1 ratio.

activity, and DNA content in femoral diaphyseal and metaphyseal tissues, was prevented by Zn supplementation of 100 mg/kg for 2 weeks.[33] Although no information about Zn-mediated cardiac protection from diabetes has been documented, a protective effect by Zn supplementation against cardiac injury from other oxidative stresses has been described in both human and animal studies.[17,21,28] The present study provides the first evidence that Zn supplementation can prevent diabetic cardiomyopathy.

We supplied Zn to diabetic mice for only 3 months after diagnosis of diabetes for the following 2 reasons: (1) Diabetic cardiomyopathy, although a chronic symptom, is believed to be secondary to early cardiac injuries caused by diabetes[22,34,35]; and (2) we have found that a temporal and recoverable cardiac dysfunction could be observed in diabetic mice at an early stage ($\approx$2 weeks),[13,22] which was also noted in diabetic rats.[36] However, irreversible cardiac dysfunction may begin at $\approx$3 months after streptozotocin treatment.[14,2] In this first study, therefore, we tried to determine whether induction of cardiac MT for the first 3 months, starting immediately after the onset of diabetes,

prevents the development of diabetic cardiomyopathy at a later stage. The results showed that induction of cardiac MT by Zn supplementation (Figure 4) was accompanied by a significant prevention of diabetic cardiomyopathy examined at 6 months after the onset of diabetes (Figures 2 and 3, Table).

Until the present study, the role of MT induction in the protective action of Zn supplementation against diabetic complications was not clear because MT had not been measured in the aforementioned experiments.[30–33] However, evidence that MT induction by Zn supplementation plays a major role in protection against nondiabetic injuries has been demonstrated.[17–20,28] We have also shown that Zn administration caused a marked MT synthesis in rabbit lymphocytes and in cultured human neuronal cells that provided a significant resistance to radiation-induced DNA damage and cell death.[37–39] In the present study, supplementation with Zn for 3 months provided a significant protective action on diabetic cardiomyopathy along with the significant induction of MT.

Direct cytotoxic protection by Zn was excluded by the finding in vitro that low-dose Cd conferred protection similar to that of
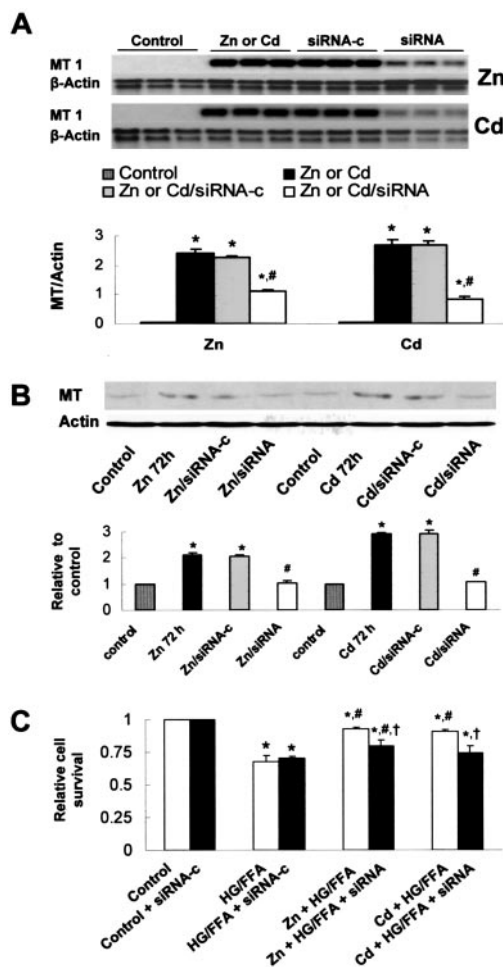
Figure 7. Preventive action of Zn or Cd pretreatment against HG/FFA-induced cytotoxicity was abolished by silencing MT mRNA expression by the MT siRNA technique. Cardiac cells (H9C2) were exposed to Zn (50 μmol/L) or Cd (1.0 μmol/L) for 24 hours, which was followed by replacement with fresh medium containing either MT siRNA or MT siRNA-control (final concentration, 1 μg/mL) for 6 hours. After this incubation, some cells underwent immediate RNA extraction for MT mRNA measurement by Northern blotting (A), and others were bathed in fresh medium for an additional 42-hour incubation and then harvested for MT protein measurement by Western blotting (B). For measurements of cell viability (C), cells were treated under the same conditions as for Western blotting (total of 72 hour of treatment) but were then exposed to an additional 24 hours of HG/FFA treatment and compared with appropriate controls. Three experiments with at least 3 samples per group were performed. *$P<0.05$ vs control; #$P<0.05$ vs HG/FFA only; †$P<0.05$ vs Zn+HG/FFA or Cd+HG/FFA. HG indicates 22.5 mmol/L glucose; FFA, 50 μmol/L palmitate bound to BSA in a 5:1 ratio.

Zn in a cell model of diabetes. Cd is a toxic metal at high doses and a potent inducer of MT.[7,8] We have previously shown that pretreatment with low-dose Cd, at which no cytotoxic effect was observed in the cultured cells, significantly induced MT synthesis in neuronal tissues and cells, leading to a significant protection from ionizing radiation.[37–39] Furthermore, when MT expression was silenced with MT siRNA, the preventive effect of pretreatment with Zn or low-dose Cd was abolished (Figure 6 and Figure 7). We therefore conclude that the significant protection of cardiac cells from HG/FFA by pretreatment with Zn or low-dose Cd is mediated by MT induction.

The in vitro conditions used to confirm a direct role for MT in protection by Zn supplementation do not exactly represent the in vivo conditions. Therefore, in addition to induction of MT, other mechanisms that may contribute to the protective effects of Zn need to be considered. These include the roles of Zn as a nutrient and as an antioxidant. As an essential trace metal, Zn is a constituent of >300 enzymes in the human body.[28] Zn is required for normal insulin secretion and function, and Zn has an insulinlike function to stimulate glucose uptake.[8,40–42] However, diabetic patients are usually Zn deficient,[43,44] as we observed in diabetic mice (Figure 5B and 5C). Decreased Zn may cause dysfunction of certain key proteins[45]; therefore, supplemented Zn may modify these key protein functions. Because Zn acts as a potent antioxidant,[28,46] Zn supplementation may enhance the antioxidant capacity of diabetic subjects or prevent diabetes-induced oxidative damage directly through its antioxidant action or indirectly through its binding and stabilizing of cellular membranes against lipid peroxidation.[30–33]

The safety of chronic supplementation with Zn is important in consideration of the efficiency of its preventive effect. The innovation of the present study is to take a clinically feasible medication (ie, Zn) into an experimental animal setting to explore a novel therapeutic application to diabetic complications. Supplementation with Zn has been extensively used clinically without significant toxic side effects; however, long-term excess intake of Zn has occasionally caused Cu deficiency and anemia.[47–49] For example, supplementation with Zn at 100 mg/d for 5 years[49] or 1000 to 2000 mg/d for 1 year[47,48] caused significant but reversible anemia. Cu deficiency is also a common risk factor for cardiomyopathy.[23] As observed in the present study, supplementation with Zn for 3 months caused a significant decrease in hepatic Cu level in nondiabetic mice but not in diabetic mice (Figure 5). Although there was no significant cardiac toxic effect of 3 months of Zn supplementation, as examined by histology and fibrosis (Figure 2 and Figure 3), Zn supplementation did cause mild cardiac dysfunction, as shown by a reduced elevation of +dP/dt$_{max}$ after catecholamine stimulation with isoproterenol, but this effect was not observed in diabetic mice (Table). Diabetes caused a significant Zn and Cu deficiency; therefore, Zn supplementation would provide a protective effect on cardiac function rather than causing further Zn and Cu deficiency (Figure 5). These results suggest that Zn may have divergent effects on cardiac mechanical function depending on the basal Zn and Cu levels. In addition, Zn supplementation followed by a small dose of Cu supplementation was found to have no effect on Cu status in healthy men.[50] Therefore, whether Zn supplementation will confer significant therapeutic benefits for diabetic patients, especially under Zn-deficient condition, is worthy of further exploration.

A few limitations of the present study should be mentioned. The first limitation is the use of the diabetic mouse model because mouse hearts are too small to measure metals after other variables are measured. Use of rats and other larger animals will overcome this limitation. The second limitation is that only the streptozotocin-induced diabetes model was examined. The last limitation is that only 1 dose of Zn was used for the present study. Therefore, this preventive effect of Zn supplementation should be further tested in other animal models such as nonobese diabetic mice and spontaneous insulin-dependent diabetic rats,

Effects of Chronic Zn Supplementation on Cardiac Function at Baseline and After Isoproterenol Stimulation in Nondiabetic and Diabetic Mice

	Control (n=8)	Control/Zn (n=5)	Diabetes (n=6)	Diabetes/Zn (n=10)
Baseline				
Heart rate, mean (SD), bpm	370.6 (39.5)	398.3 (49.8)	355.3 (34.8)	404.2 (57.4)
SBP, mean (SD), mm Hg	93.1 (7.8)	90.3 (6.8)	92.9 (8.4)	89.0 (5.0)
DBP, mean (SD), mm Hg	66.5 (7.1)	66.3 (6.2)	67.4 (8.1)	63.7 (5.6)
LVP, median (range), mm Hg	95.3 (81.9 to 100.8)	86.5 (80.7 to 96.1)	82.2 (76.6 to 83.8)*	89.4 (77.9 to 97.1)*‡
LVEDP, median (range), mm Hg	5.6 (3 to 8.1)	6.2 (3.4 to 13.3)	6.2 (5.2 to 8.1)	2.8 (1.1 to 6.5)*‡
+dP/dt$_{max}$, median (range), mm Hg/s	4723.3 (3776.3 to 7009.2)	4634.9 (3150.4 to 5144.1)	3879.2 (3556.7 to 4069.6)*	4921.4 (3107.4 to 6304.9)‡
−dP/dt$_{min}$, median (range), mm Hg/s	4948.3 (3559.7 to 6282.2)	4135.5 (2732.6 to 4878.3)	3700.9 (3463.3 to 3941.6)*	4471.9 (2884.9 to 5363.9)‡
τ, median (range), ms	15.9 (12. to 19.2)	16.2 (15.4 to 16.3)	18.9 (17.1 to 20.8)†	14.4 (12.0 to 20.0)‡
After isoproterenol stimulation				
Heart rate, mean (SD), bpm	533.9 (14.7)	543.3 (52.07 to 563.5)	413.4 (40.3)*†	536.6 (57.8)‡
% of baseline, mean (SD)	145.5 (15.6)	137.8 (13.8)	117.5 (17.9)*	134.6 (20.2)‡
LVP, median (range), mm Hg	104.6 (102.4 to 138.9)	91.6 (78.4 to 96.4)	88.9 (66.6 to 98.6)*	95.3 (84.2 to 105.8)
% of baseline, median (range)	105.9 (103.8 to 146.1)	100.3 (90.6 to 108.6)	108.0 (86.8 to 120.7)	107.3 (101.3 to 117.0)
LVEDP, median (range), mm Hg	0.4 (−0.2 to 3.0)	0.4 (0.1 to 7.5)	3.8 (2.3 to 7.1)*	1.0 (−1.0 to 2.5)‡
% of baseline, median (range)	8.3 (−3.8 to 37.1)	12.7 (3.0 to 120.2)	68.4 (39.5 to 109.1)*	21.0 (−92.4 to 91.2)‡
+dP/dt$_{max}$, median (range), mm Hg/s	7380.5 (5726.0 to 8825.6)	5711.1 (5027.6 to 7033.0)*	4298.9 (3590.9 to 4865.1)*†	7443.7 (5605.0 to 10040.5)†‡
% of baseline, median (range)	151.4 (122.6 to 184.3)	141.0 (120.7 to 159.6)	112.7 (92.7 to 119.5)*†	158.2 (134.9 to 223.7)‡
-dP/dt$_{min}$, median (range), mm Hg/s	5018.9 (4567.9 to 6489.6)	4129.6 (3230.8 to 4758.5)*	4159.2 (3387.0 to 4928.6)*	4537.0 (3241.9 to 6167.3)†‡
% of baseline, median (range)	113.9 (83.8 to 139.6)	99.9 (95.7 to 124.2)	108.9 (95.1 to 134.9)	103.1 (76.6 to 131.3)
τ, median (range), ms	14.7 (13.2 to 15.3)	13.8 (13.2 to 17.2)	16.7 (15.5 to 26.7)*	14.2 (12.0 to 16.1)‡
% of baseline, median (range)	89.4 (77.5 to 122.8)	85.8 (84.2 to 106.1)	98.0 (80.5 to 128.4)	99.6 (71.8 to 109.6)

SBP indicates systolic blood pressure; DBP, diastolic blood pressure; LVP, LV peak systolic pressure; LVEDP, LV end-diastolic pressure; +dP/dt$_{max}$, maximum rate of rise in intraventricular pressure during ventricular contraction; −dP/dt$_{min}$, maximum rate of decrease in intraventricular pressure during isovolumetric relaxation; and τ, time constant duration relaxation.

*$P<0.0125$ vs control; †$P<0.0125$ vs control/Zn; ‡$P<0.0125$ vs diabetes group.

and an optimal protocol of Zn supplementation should be determined to more efficiently prevent diabetic cardiomyopathy. Thus, further studies are warranted.

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CLINICAL PERSPECTIVE

Diabetic cardiomyopathy may be directly related to oxidative stress. Our previous studies showed that transgenic mice that overexpress cardiac-specific metallothionein (MT), a potent antioxidant, are highly resistant to diabetes-induced cardiomyopathy. Zinc is the major metal that binds to MT under physiological conditions and is an effective inducer of MT. The present study therefore explored whether zinc supplementation can protect against diabetic cardiomyopathy through cardiac MT induction. The present study provided evidence that zinc supplementation to diabetic mice significantly prevented the increases in cardiac morphological impairment, fibrosis, and dysfunction observed in diabetic mice without zinc supplementation. Zinc supplementation also induced a significant increase in cardiac MT expression. When MT expression was silenced with the use of MT small-interfering RNA in cultured cardiomyocytes, the preventive effect of pretreatment with zinc was abolished. These results suggest that the prevention of diabetic cardiomyopathy by zinc supplementation is predominantly mediated by an increase in cardiac MT. This is a very important finding with a high potential for clinical application to prevent diabetic cardiomyopathy. Because MT is an efficient antioxidant and is highly inducible in the heart, and also because zinc is an effective MT inducer and has been used clinically without significant toxicity, we hypothesize that zinc supplementation will be a feasible approach to clinical application for the prevention of diabetic cardiovascular disease after further studies on the optimal dose levels and supplemental means.